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Research Article

Antibacterial activity of thin films TiO_2 doped with Ag and Cu on Gracilicutes and Firmicutes bacteria

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Abstract

This article aims to explore the antibacterial activity of thin films of TiO_2 doped with Ag and Cu using two types of Gram-negative and Gram-positive test bacteria with clinical significance (Gracilicutes and Firmcutes bacteria). The thin films (thickness of about 60 nm) were deposited on glass substrates by radio frequency magnetron co-sputtering (r.f. power of 50 W) of TiO_2 target with Ag and Cu pieces on its surface in an Ar atmosphere (0.8 Pa) without heating during the deposition. The total surface area of the Ag was 60 mm^2 and that of the Cu was 100 mm^2. *Bacillus cereus, Staphylococcus epidermidis, Salmonella enterica, Escherichia coli* and *Pseudomonas sp.* were used as test strains. The antibacterial actvity of the films was evaluated by the classical Koch's method and optical density measurements. The bactericidal effect was established at different time points between 30 min and 90 min for *Pseudomonas sp.* and *S. enterica*. The Firmicutes bacteria *B. cereus* and *S. epidermidis* were killed at the 4^{th} and 8^{th} hour of the treatment, respectively. The effect on *E. coli* was bacteriostatic until the 10^{th} hour. The results were confirmed by assessment of the bacterial dehydrogenase activity. The studied thin films of TiO_2 co-doped with Ag and Cu have a potential for application as antibacterial coatings.

Keywords

TiO_2 thin films, bactericidal effect, Gram-positive and Gram-negative bacteria, clinically significant strains

Introduction

The antibacterial effect of the nanomaterials is due to different processes based on their surface biochemical functionality, partial dissolution of their components and mechanical antibacterial effect of their surface morphology. The utilisation of nanostructured materials as antimicrobial agents depends on their high surface to volume ratio. TiO_2 is a widely used material for scientific studies and different applications in clinical practice due to its biocompatibility and high stability at chemical treatments and environmental conditions. The modification of the TiO_2 band gap through doping with different chemical elements does not affect its ultraviolet light activity and increases the photocatalytic activity in sunlight. It is known that transition metals (W, V, Ag, Cu etc.) or non-metals (S, C, N_2) doped TiO_2 has a lower band gap than pure TiO_2 and can be used to increase the production of reactive oxygen species (ROS) under visible light irradiation in solutions containing undesired pollutants Fujishima et al. (2008), Huang et al. (2012). Doped with silver, TiO_2 nanoparticles (NPs) prepared by sol-gel technique were successfully applied for deactivation of different microorganisms (*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus*). A concentration dependence was established for the bacterial deactivation with Ag content in TiO_2 thin films with a quantity of 1, 3, 5 and 7 μg/ml Bahadur et al. (2016). Wong et al. (2015) reported increasing the visible-light response and durability of silver nanoparticles impregnated in TiO_2:(N) thin films against pathogens like *E. coli, Streptococcus pyogenes, S. aureus* and *Acinetobacter baumanii*.

TiO_2 porous coatings with different Ag concentration (0, 0.95, 1.36 and 1.93 wt%, respectively) prepared through combining magnetron sputtering with micro-arc oxidation were presented by Zhang et al. (2014). The interaction of the coatings with bacterial culture is characterised by different rates of Ag dissolution - initially high rate followed by a period of low rate. This process provided relatively long-term antibacterial activities and met the clinical requirements. No cytotoxic effect is found for the surfaces with Ag concentration lower than 0.95 wt%.

Heidenau et al. (2005) and Haenle et al. (2010) demonstrated that Cu induces bacterial cell toxicity without decreasing the cell biocompatibility. The osteoblastic adhesion, spreading, early proliferation and late differentiation on Cu-TiO_2 coatings were significantly enhanced due to the inhibitory effect of Cu and existence of a suitable porous nano-structured surface Han et al. (2008), Zhao et al. (2009). Excellent antibacterial properties of Cu-doped TiO_2 coatings were reported by Raffi et al. (2010). They accept that the reason for the antibacterial property of Cu NPs is their adhesion on to the bacterial surface due to their opposite charge with respect to bacteria. When they encounter each other, the reduction reaction occurs at the bacterial cell wall, resulting in formation of cavities or pits.

According to Zhu et al. (2013), Wu et al. (2014), Cu as an essential trace element participating in a variety of metabolic activities in living organisms, might be a more effective antimicrobial agent than Ag due to its low cytotoxicity. According to Norambuena et al. (2016), amongst metals with antibacterial properties, copper has shown superior *in vitro* antibacterial performance while maintaining an acceptable cytotoxicity profile. A thin film containing copper could prevent early formation of biofilm which limits periprosthetic infections. TiCuO containing 20%, 40% or 80% copper prepared through high-power impulse magnetron sputtering (HiPIMS) showed an increased biofilm and planktonic cell density reduction.

Zhang et al. (2016) used Micro-Arc Oxidation synthesised TiO_2 coatings doped with different doses of Cu^{2+} (0 - 1.93 wt%) on *S. aureus*. Compared to Ti, the adhesion and proliferation of fibroblast can be significantly enhanced on the TiO_2 coating with 0.67 wt% Cu, while these processes are greatly inhibited with 1.93 wt% Cu. Cu^{2+}-doped TiO_2 coatings show good antibacterial properties, including contact-killing and release-killing of *S. aureus*.

Rtimi et al. (2014) compared the antibacterial effect of Ag and Cu surfaces as a functionalisation of polyester by very thin sputtered Ag and Cu layers inducing accelerated *E. coli* killing in the dark. In the case of Ag, the most suitable sample leading to a bacterial inactivation within 2h required a sputtering time of 75 s or 150 s. The deactivation kinetics of *E. coli* on Cu-polyester was observed in the sample which was sputtered for 30 s and 60 s. It was established that, at longer HIPIMS sputtering times, the inactivation time for *E. coli* becomes shorter. A local softening of the fibres at the contact sites with Cu may play a significant role in the inactivation time of *E. coli*. These films preclude formation of biofilm of all kinds of infectious bacteria in hospitals, schools and public places.

Santhosh and Natarajan (2015) reported excellent biocidal characteristics for the epoxy resin containing TiO_2 doped with Ag^+. They found significant inhibition of the biofilm development by two pathogenic bacteria, *Staphylococcus aureus* and *Escherichia coli K-12.*

He et al. (2017) carried out a review of micro-arc oxidation assisted magnetron sputtering (MS) of Ag, Cu and ZnO to endow Ti-implant materials with antibacterial properties.

To summarise, no detailed research has been reported for antimicrobial activity of TiO_2 thin films doped with Ag and Cu and deposited through r.f.magnetron co-sputtering. This is the reason to study the antibacterial effect of these films and compare their influence on Gracilicutes and Firmicutes bacteria.

Material and methods

Preparation of thin film coated glass substrates

The thin films were deposited on glass substrates by r.f. magnetron co-sputtering (13.56 MHz; r.f. power of 50 W; in Ar atmosphere at 0.8 Pa) of the TiO_2 target with Ag and Cu

pieces on the surface in its maximum erosion zone without heating during the deposition. The total surface area of Ag (4 similar pieces) was 60 mm^2 and that for Cu (4 similar pieces) was 100 mm^2. The size of the glass substrates was 20 x 25 mm, preliminarily treated by a solution of H_2SO_4 : H_2O_2 (1:1) to ensure the adhesion of coating. The thickness of the films was about 60 nm, measured by a Taylor Hobson profilometer. The optical properties of the films were analysed through transmittance and reflectance measurements by a Shimadzu 3600 spectrophotometer.

Bacterial strains

The following bacteria were used in this study: *E. coli* ATCC 10536, *Pseudomonas putida* ATCC 12633, *Bacillus cereus* ATCC 7050, *Staphylococcus epidermidis* ATCC 12228 and *Salmonella enterica* serotype *choleraesius* DSMC 4224; all supplied by the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC).

Antimicrobial test

Diffusion assay

A diffusion assay was used for fast screening. The test bacterial strains were grown in the most suitable growth medium for each species at 180 rpm for 18 h. The microbial density of 0.5 - 0.8 was determined according to McFarland. The aliquots of 100 µl microbial suspension were randomly spread on a solid nutrient medium (Conda, Spain or ISO 10712 for *Pseudomonas*) and the glass matrices of the investigated material were added. The plates were left for 20 h at 4 - 6°C to afford diffusion of the nanoparticles and after, theywere cultivated for 24 h at 25°C for *P. putida* and 37°C for all other bacteria. The sterile zones formed around the samples were measured in mm ($\pm$ 0.5).

Disinfection time

Determination of the disinfection time was conducted for 24 h in a light-dark regime with illumination for 15 min during the sampling in Corning® Costar® TC-Treated six-well plates. Two wells were used for the control as bare glasses (without thin films) and the other four wells with thin film coated glass substrates were placed and sterilised by 30 min UV-irradiation. In the well with the blank control, a sterile nutrient liquid medium was added over the thin sterile film without bacteria to measure the thin film dissolution and light absorption of the liquid (Nutrient broth, Conda, Spain or ISO 10712 for *Pseudomonas putida*). In the remaining 5 wells, a liquid culture medium inoculated with a test microorganism with initial concentration (OD 0.001) of overnight bacterial culture in the exponential phase was added. The inoculum and medium quantity in all variants were the same for all samples and controls at the beginning of the experiment. The optical density was measured every hour with a Zeiss spectrophotometer at λ = 610 nm. Also, the determination of surviving bacteria was studied through the classical Koch's method. Every 2 h, consecutive decimal dilutions were prepared for determination of the bacterial amount

of the samples and the controls. After plating of 100 µl bacterial suspension on a solid nutrient medium without thin films and cultivation at 25°C for *P. putida* and 37°C for all other bacteria, counting of the bacterial colonies was conducted.

The maximum growth rate (µ) of the control and the samples was calculated through the equation:

$\mu = (\lg x - \lg x_0)/(\lg 2)(t-t_0) = (\lg N - \lg N_0)/0{,}3(t-t_0)$ (1),

where:

x_0, N_0 - the initial quantity of the bacteria, evaluated by classical Koch's method

x, N - the final quantity of the bacteria, evaluated by classical Koch's method

$t - t_0$ - incubation time.

Assessment of the dehydrogenase activity

Iodonitro-tetrazolium chloride (INT) was used for assessment of the dehydrogenase activity inhibition. It is known that INT has a higher sensitivity to dehydrogenase enzymes than triphenyl tetrazolium chloride (TTC), but TTC is more resistant to Cu ions in the suspensions. This was the reason to use both reagents INT and TTC for its parallel determination. No differences were detected between them. The optical density of the stained supernatant was measured at λ = 465-480 nm. The control is usually stained as bright-red and the samples as yellow or light red colour depending on the inhibition of enzyme activity. The optical density results were recalculated using a standard value for the amount of colourant obtained and the amount of protein determined as described by Groudeva et al. (2006). Inhibition of total dehydrogenase activity was calculated as a decrease in the enzyme activity as a percentage of the control in formazin units/mg of protein.

SEM observations

A Scanning Electron Microscope, JOEL model JSM 5510 (Japan) was used for observation of the bacterial surface morphology on the thin films.

Atomic Flame Absorption Spectrometry

Atomic Flame Absorption Spectrometry (Perkin Elmer Aanalyst 400) was used for determination of Ag and Cu in the liquid and control medium as described by Fathi et al. (2009). The total metal content (nanoparticles and ions) was determined at the 24th hour, as follows:

$C_{Me} = (Ko-S)/A.CFU.mL^{-1}$ (2),

where:

Ko - dissolved NPs and ions from control (thin film in nutrient medium without bacteria),
S - dissolved NPs and ions from sample (thin film in nutrient medium with bacteria),
A - atomic mass of the studied metal,
$CFU.mL^{-1}$ - Colony Forming Units (CFU) initial bacterial quantity in one ml of culture medium.

The values of the dissolved and permanently associated with the organic molecules Ag nanoparticles and ions were accounted. The results were represented as mol/cell.

Results

Inhibitory effect in static conditions

The result from the diffusion assay test have shown that the inhibition zone was different for the test bacteria: 11 mm for *E. coli* and *S. epidermidis*, 12 mm for *B. cereus* and 13-14 mm for *Pseudomonas sp.* It can be concluded that *E. coli* and *S. epidermidis* are the most resistant to the tested films.

Disinfection time

The disinfection time of the studied thin films was determined for all tested bacteria during cultivation in the liquid medium.

The disinfection time of the coating to Gram-positive pathogen, *B. cereus* is presented in Fig. 1. It is evident that the bacterial growth in the presence of TiO_2:Ag:Cu thin films was strongly inhibited. Both methods used to obtain the growth curve demonstrate similar dependencies. The number of bacteria in a logarithmic scale sharply decreased at the 4^{th} hour and the optical density dropped at the 3^{th} hour from the beginning of the experiment, which determined the process of disinfection. There are no surviving cells till the 48^{th} hour, which also suggests the killing effect of the thin film on the spores of *Bacillus cereus* strain.

The effect of tested thin films on the other Firmicutes bacteria, like *S. epidermidis* can be seen in Fig. 2a and Fig. 2b. In this case, a slower bactericidal effect and no surviving cells after the 24^{th} hour were observed. The result could be due to the formation of a biofilm on the surface, typical for this kind of bacteria and lower growth rate (0.2 in comparison with the 0.4 for *Bacillus*). As is well known, the cells in a biofilm are more resistant to the environmental factors.

Fig. 3 demonstrates the bactericidal effect of the same thin films TiO_2:Ag:Cu on *P. putida* growth, determined by the cultivation method Fig. 3a. The results obtained by the optical density measurements at λ = 610 nm are shown in Fig. 3b. The bactericidal effect on *Pseudomonas* strain occurred faster and the result was visualised as a morphological destruction of bacterial cells by a Scanning Electron Microscope (JOEL JSM 5510). The damaged cells can be viewed in two optical magnifications in Fig. 4.

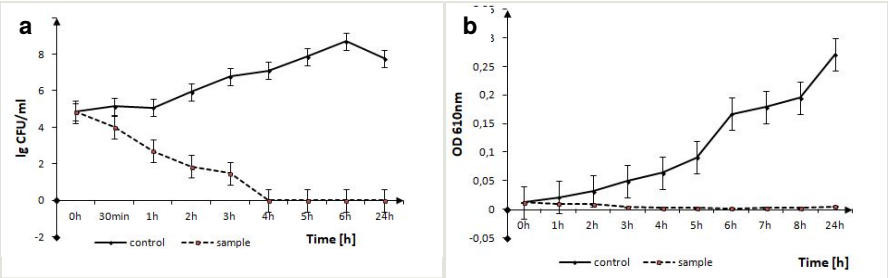

Figure 1.

B. cereus growth in the presence of TiO_2:Ag:Cu thin films. The solid line represents the bacterial quantity for the control without TiO_2:Ag:Cu film and dashed line represents the quantity of surviving cells in sample with TiO_2:Ag:Cu film, both in logarithmic scale.

a: Koch's method

b: optical density measurements

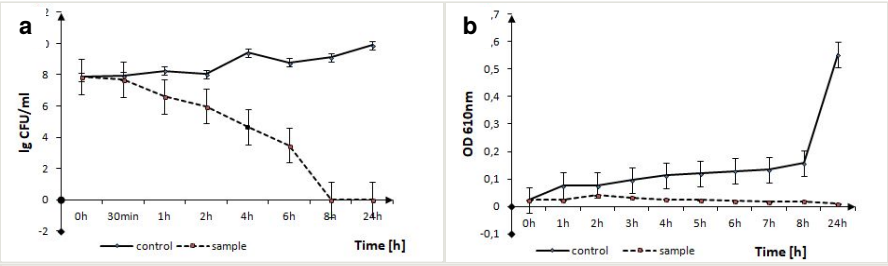

Figure 2.

S. epidermidis growth in the presence of TiO_2:Ag:Cu thin films. The solid line represents the bacterial quantity for the control without TiO_2: Ag: Cu film and the dashed line represents the quantity of surviving cells in the sample with TiO_2:Ag:Cu film.

a: Koch's method

b: Optical density measurements

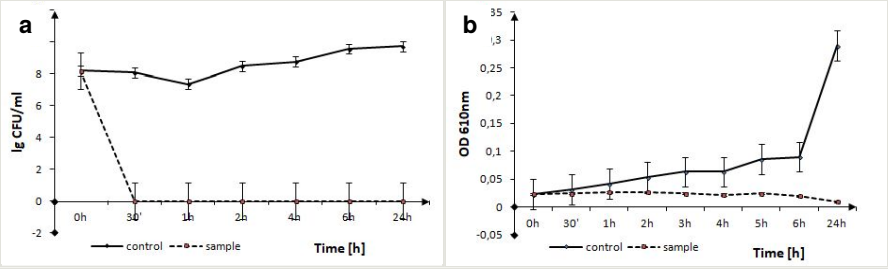

Figure 3.

P. putida growth in the presence of TiO_2:Ag:Cu thin films. The solid line represents the bacterial quantity for the control without TiO_2:Ag:Cu film and the dashed line represents the quantity of surviving cells in the sample with TiO_2:Ag:Cu film, both in logarithmic scale.

a: Koch's method

b: optical density measurements

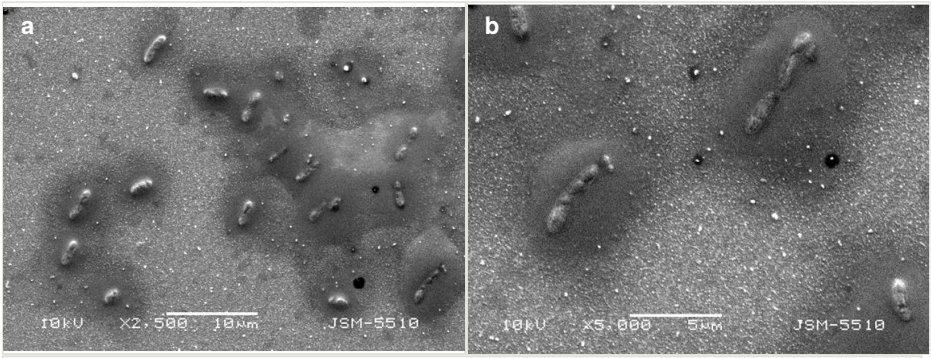

Figure 4.

The image of *P. putida* cells on thin TiO_2:Ag:Cu film by Scanning Electron Microscope with different magnifications.

a: x2500
b: x5000

The influence of nanostructured thin films TiO_2:Ag:Cu on the growth of *S. enterica* is represented in Fig. 5a and Fig. 5b. The bactericidal effect on Gracilicutes bacteria (*S. enterica* and *Pseudomonas sp.)* is faster than the bactericidal effect on the Firmicutes, which supposes the higher sensitivity to the nanocomposite material. The effect is not the same on *E. coli* - the thin film has only a bacteriostatic effect on it. Strong retention in the bacterial growth was observed from the beginning until the 10^{th} hour of the experiment. The results for *E. coli* are presented in Fig. 6.

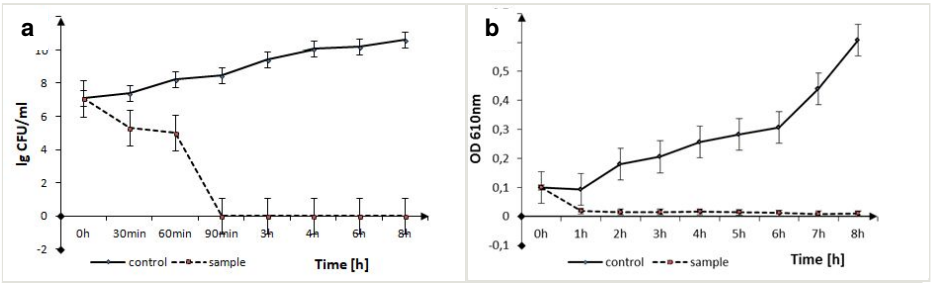

Figure 5.

S. enterica growth in the presence of TiO_2:Ag:Cu thin films. The solid line represents the bacterial quantity for the control without TiO_2:Ag:Cu film and the dashed line represents the quantity of surviving cells in the sample with TiO_2:Ag:Cu film in logarithmic scale.

a: Koch's method
b: optical density measurements

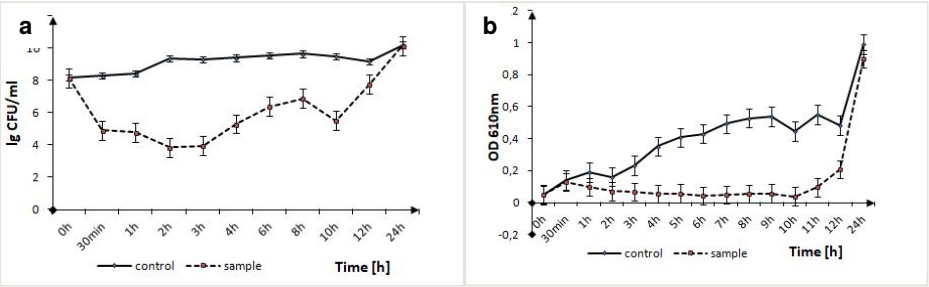

Figure 6.

E. coli growth in the presence of TiO_2:Ag:Cu thin films. The solid line represents the bacterial quantity for the control without TiO_2:Ag:Cu film and the dashed line represents the quantity of surviving cells in the sample with TiO_2:Ag:Cu film in logarithmic scale.

a: Koch's method
b: optical density measurements

Atomic Flame Absorption Spectrometry

The bactericidal concentration of Ag and Cu (nanoparticles and ions) in solution was measured for the representatives of two types of the tested bacteria - Firmicutes and Gracilicutes. The values for blank control (organic nutrient medium with a thin film without bacteria) were determined as 5.2 mg/l for Ag and as 4.2 mg/l for Cu. For the samples with bacteria (*B. cereus*) and thin films, these values were 4.8 mg/l for Ag in organic growth medium and 3.6 mg/l for Cu also in organic medium; the difference between the control and the sample is the amount engulfed by the *Bacillus* cells (10^5 CFU/ml) - 0.6 mg/l Cu and 0.4 mg/l Ag.

In the synthetic culture medium ISO 10712, the content of dissolved Ag was 1.04 mg/land Cu was 2.4 mg/l in the sample with 10^8 CFU *P. putida* per ml.

Discussion

In this study, five bacterial strains were exposed to TiO_2:Ag:Cu thin films. The results indicated that the tested materials have a bactericidal effect on the bacterial cells in different time periods.

The bactericidal effects of TiO_2:Ag:Cu was evident after 30 min and 90 min of exposure of *P. putida* and *S. enterica* determined by the Koch's method. Only on *E. coli*, known as a highly adaptable bacterial species, the effect was bacteriostatic. However, a strong retention until the 10^{th} hour of the treatment was observed. For the tested Firmicutes bacteria, the bactericidal effect was observed at the 4^{th} and 8^{th} hours for *B. cereus* and *S. epidermidis* respectively. This effect is within the time limits which do not allow biofilm formation. As reported by Thuptimdang et al. (2015) for monitoring the changes in bacterial activity during the biofilm formation, it is necessary to measure the bacterial quantity every 3 h for the first 24 h and every 12 h between 24th and 72^{th} hours. In this case, the

inactivation of the bacteria occurred much faster. Scanning electron microscope images reveal a rough cell envelope instead of a smooth *Pseudomonas* capsule and leakage of the cell content around the cells (dark zones) which proved the destruction of the cell envelope. Similar effects were expected for the other test bacteria. The damage of the cell envelope leads directly to the leakage of cell content materials causing cell death. This proves the mechanical demolition by nanoparticles and chemical destruction by ROS, as was established by other authors Sondi and Salopek-Sondi (2004), Zavilgelsky et al. (2011), Zhang et al. (2014).

At the end of the experiment, only 2.8% retention in the maximum growth rate at the 24^{th} hour for *E. coli* was observed. In contrast, all the other bacterial strains were completely deactivated. The maximum growth rate counted on the controls data obtained by Koch's method (test bacterial strains without nanomaterials) were 0.404, 0.279, 0.214, 1.472 and 0.283 for *B. cereus, S. epidermidis, P. putida and S. enterica* respectively.

The toxic effect was confirmed by an assessment of the bacterial dehydrogenase activity. As is well known, this activity is proof of normal cell metabolism and its inhibition is due to destruction of the bacterial cell wall and membrane. The stress response in the treated *E. coli* cells was the dehydrogenase acivity increase in the sample in comparison to the control. The other bacterial strains, in contact with the *E. coli*, have shown no enzyme activity when tested with INT and TTC. Only *E. coli* retains the dehydrogenase activity at the 24^{th} hour; the formazan quantity in the sample measured was OD_{470} = 0.804 compared to the OD_{470} = 0.671 in the control and the calculated enzyme activity in the sample was 0.253 FNU (Formazin Nephelometric Units), compared to the control (0.08 FNU). The bacteriostatic effect and the adaptation of *E. coli* to the tested nanomaterials could be explained by the ability of this bacterial species to activate special transport pumps for export of the metal ions out of the cell. This fact is the mechanism of stress response as has been reported by Stoyanov et al. (2003). They found that a special transport pump coded by CopA gene of *E. coli* can control cytoplasmic silver, in addition to copper content.

The tested material demonstrated bactericidal concentration on *P. putida* at 3.77 x 10^{-7} mol/cell for Cu and 9.64 x 10^{-11} mol/cell for Ag. For the Firmicutes representative *B. cereus*, the toxic effect was observed at 3.71 x 10^{-8} mol/cell for Ag and 9.44 x 10^{-6} mol/cell for Cu. These results confirmed that the Gracilicutes bacteria are more sensitive to dissolved Ag and Cu from the studied thin films.

The results from the atomic absorption spectrometry proved a partial dissolution of metal atoms and ions from the tested thin films. Probably as a result of the faster penetration of Ag and Cu atoms in the cell, they cause a quicker toxic effect on the Gracilicutes bacteria. Moreover, due to the established low concentration of Ag and Cu in our experiment, low cytotoxicity to human cells can also be expected. A concentration of 5 µg/ml Ag nanoparticles (NPs) affects the epithelial cell line, according to Comfort et al. (2011).

The neurotoxicity of Ag NPs with an average size of 15 nm at a concentration of 10 µg/ml for 24 h was investigated using the dopaminergic neuronal cell line PC12 by Wang et al. (2009). From the results of this study, it can be inferred that the silver is involved not only in

the oxidative stress, but also in alteration of the enzymatic functions which play an important role in the dopamine depletion.

The effects of the tested thin films depend on the structure of the bacterial cell wall Allaker (2010), Wang et al. (2012). In these cases, the peptidoglycan thickness plays an important role in the interaction with the nanoparticles, but this effect might be modulated by the other features of the environment, such as pH, the metal structure or the carrier Allaker (2010), Seil and Websters (2012), Wang et al. (2012). A similar effect has been observed in the study of Spacciapoli et al. (2001), Sondi and Salopek-Sondi (2004), Taglietti et al. (2012). A ten times higher concentration was obtained for Ag for *Bacillus* cells in neutral pH=7.0 than that of *Pseudomonas* at slightly acidic pH=6.0 and a longer time for the bacterial contact killing - 4 h for *Bacillus* in contrast with *Pseudomonas sp.* - only 30 min for their inactivation. According to Foster et al. (2010), the mechanisims of action include a release of more ionic silver than Ag^0 atoms.

The study of Santhosh and Natarajan (2015) indicates that the effective dispersion and the optimum release of biocidal agents was responsible for the anti-biofilm activity of the surface. Quiñones-Jurado et al. (2014) reported that the surface plasmon resonance of Ag NPs plays an important role in dependence on the NPs' size and increases the bactericidal effect of Ag/TiO_2 nanocomposite. They supposed that, under visible light, the reactivity of TiO_2 cannot be amplified when it supports Ag NPs which have an inactive photocathalytic surface. In this case, the reactivity of Ag and Cu metals is increased probably due to the synergistic effect of Ag and Cu in the TiO_2 matrix. Also, nanoparticles of Cu possess a high oxidative redox potential leading to a fast bacterial loss of viability. The Cu ions entering the bacterial cytoplasm bind S, N and COO- electron donor negative groups of the cell wall.

Conclusions

The antibacterial activity of TiO_2 thin films doped with Ag and Cu using two types of Gram-negative and Gram-positive test bacteria with clinical significance (Gracilicutes and Firmicutes bacteria) were tested. The antibacterial activity of the thin films was evaluated by the classical Koch's method and optical density and dehydrogenase activity measurements. The bactericidal effect was established at different time periods between 30 min - 90 min for *Pseudomonas sp*. and *S. enterica*. The Firmicutes bacteria *B. cereus* and *S. epidermidis* were killed at the 4^{th} and 8^{th} hours of the treatment respectively. The effect against *E. coli* was bacteriostatic until the 10^{th} hour. The studied TiO_2:Ag:Cu thin films have potential for application as antibacterial agents on clinically significant bacterial strains. For the future perspective, other technological variants with similar combinations will be tested.

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